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LESA MS imaging of heat preserved and frozen tissue: Benefits of multi-step static FAIMS

Rian L. Griffiths¹, Anna L. Simmonds¹, John G. Swales², Richard J. A. Goodwin² and Helen J. Cooper^{1*}

1. School of Biosciences, University of Birmingham, Edgbaston, B15 2TT, U.K.
2. Pathology, Drug Safety & Metabolism, IMED Biotech Unit, AstraZeneca, Darwin Building, Cambridge Science Park, Milton Road, Cambridge, CB4 0WG, U.K.

*To whom correspondence should be addressed: h.j.cooper@bham.ac.uk

Abstract

We have previously demonstrated liquid extraction surface analysis (LESA) high field asymmetric waveform ion mobility spectrometry (FAIMS) mass spectrometry imaging of proteins in thin tissue sections of brain and liver. Here, we present an improved approach which makes use of multiple static FAIMS parameters at each sampled location and allows a significant improvement in the number of proteins, lipids and drugs that can be imaged simultaneously. The approach is applied to the mass spectrometry imaging of control and cassette-dosed rat kidneys. Mass spectrometry imaging of kidneys typically requires washing to remove excess hemoglobin; however, that is not necessary with this approach. Multi-step static FAIMS mass spectrometry resulted in a six- to sixteen-fold increase in the number of proteins detected in the absence of FAIMS, in addition to smaller increases over single step static FAIMS (chosen for optimum transmission of total protein ions). The benefits of multi-step static FAIMS mass spectrometry for protein detection are also shown for sections of testes. The numbers of proteins detected following multistep FAIMS increased between two- and three-fold over single step FAIMS, and between two- and fourteen-fold over LESA alone. Finally, to date, LESA mass spectrometry of proteins in tissue has been undertaken solely on fresh frozen samples. In this work, we demonstrate that heat-preserved tissues are also suitable for these analyses. Heat preservation of tissue improved the number of proteins detected by LESA MS for both kidney and testes tissue (by between two- and fourfold). For both tissue types, the majority of the proteins additionally detected in the heat-treated samples were subsequently detected in the frozen samples when FAIMS was incorporated. Improvements in the numbers of proteins detected were observed for LESA FAIMS MS for the kidney tissue; for testes tissue fewer total proteins were detected following heat preservation, however approximately one third were unique to the heat preserved samples.

Introduction

Liquid extraction surface analysis (LESA)¹ mass spectrometry is a direct surface sampling technique which is suited to the analysis of intact proteins², lipids³ and exogenous drug compounds.⁴ We have previously shown that intact proteins can be extracted via LESA from substrates including dried blood spots on filter paper⁵⁻⁷, thin tissue sections thaw-mounted onto glass⁸ and *E. coli* colonies growing on agar⁹. Moreover, intact non-covalent protein complexes can be sampled by LESA.^{10, 11} We have recently demonstrated that LESA may be applied for mass spectrometry imaging of intact proteins and protein complexes in liver and brain tissue.^{12, 13} LESA has been described for drug imaging in numerous studies;^{4, 14, 15} imaging of both intact proteins and drugs in dosed tissue samples could prove beneficial.

An inherent challenge for LESA mass spectrometry of biological substrates is the complexity of the extracted sample. Many molecular classes may be present and extracted, potentially interfering with the detection of the analytes of interest, i.e., proteins. This challenge can be addressed by coupling LESA mass spectrometry with high field asymmetric waveform ion mobility spectrometry (FAIMS).¹⁶⁻¹⁹ FAIMS is a technique which separates gas-phase ions by exploiting differences in their mobilities in high and low electric fields. Ions are passed by a carrier gas between parallel electrodes to which an asymmetric waveform is applied, resulting in an alternating high electric field (the dispersion field, DF), and (opposite polarity) low electric field. As a result of their differential mobilities, the ions will stray from their original trajectory. This deviation in trajectory can be corrected by superposition of a dc compensation voltage. By tuning the compensation field (CF), it is possible to selectively transmit ions of particular differential mobility. FAIMS was first applied to the analysis of intact protein ions by Purves and Guevremont²⁰ who demonstrated that the charge state distribution observed for cytochrome C varied with FAIMS conditions. Subsequent FAIMS analysis of proteins has included separation of ubiquitin and cytochrome C protein conformers²¹⁻²⁷, as well as myoglobin²⁸, bovine serum albumin²⁹ and β 2-microglobulin³⁰.

The coupling of FAIMS with ambient mass spectrometry was first demonstrated by Galhena et al. in their work on desorption electrospray ionisation of small molecule drugs.³¹ Porta et al. coupled FAIMS with LESA for the analysis of small molecule drugs of abuse and their metabolites.³² For protein analysis, we have shown that incorporation of FAIMS within the LESA mass spectrometry workflow results in molecular separation, improved signal-to-noise ratios and reduced chemical noise, thus increasing the numbers of intact proteins detected. LESA FAIMS protein mass spectrometry has been demonstrated for living bacterial colonies³³, dried blood spots³⁴ and thin tissue sections.³³ Similar benefits have been described for intact proteins via ambient surface

sampling techniques such as DESI and Flowprobe mass spectrometry coupled to FAIMS.^{35, 36} We have recently demonstrated LESA FAIMS mass spectrometry imaging of intact proteins in thin tissue sections of mouse brain and liver.³⁷ In those experiments, a LESA 2-D FAIMS optimisation analysis was first performed, followed by LESA static FAIMS mass spectrometry imaging. In the 2-D FAIMS analysis, the DF and CF values were varied and the optimum values for transmission of proteins were determined. These values were subsequently applied in the LESA static FAIMS mass spectrometry imaging analysis.

To date, all LESA and LESA FAIMS mass spectrometry of tissue has been performed on fresh frozen tissue. Recently, a new tissue preservation method, based on heat fixation and commercialised by Denator, has been described³⁸. The technique uses rapid conductive heating to irreversibly, thermally denature proteins thus preventing further enzymatic activity and sample degradation. It has been shown to be compatible with proteomics, preserving peptides, proteins and post-translational modifications.³⁹⁻⁴¹ Moreover, tissue treated in this manner has been shown to be suitable for matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry imaging of peptides and proteins.^{42, 43}

Here, we present an improved LESA FAIMS mass spectrometry imaging workflow, which makes use of multiple static FAIMS analyses at each LESA-sampled location, and apply the approach to the imaging of intact proteins in samples of heat preserved and frozen tissue from rat kidneys and testes. Kidneys present a particular challenge for intact protein imaging due to the high abundance of hemoglobin, a consequence of the kidney's role in blood filtration. For example, MALDI mass spectrometry imaging of kidney requires a pre-washing step to remove hemoglobin.⁴⁴ We show here that a single step static FAIMS analysis, based on optimum transmission of total protein ions, is dominated by α - and β -globin ions; however the multi-step approach significantly increases the number of proteins detected. For example, seven proteins were detected in undosed fresh frozen kidney tissue in a single-step static FAIMS analysis (DF = 270 Td, CF = 2.5 Td), compared with 55 proteins detected in the multi-step approach. Similar improvements were observed in the multi-step FAIMS analyses of testes tissue. The use of multi-step FAIMS to improve peptide detection in LC MS/MS proteomics experiments has been demonstrated previously⁴⁵⁻⁴⁷; the current work applies the approach to mass spectrometry imaging.

We also compared the results obtained from heat preserved and frozen tissue. For the kidney tissue, a greater number of proteins were detected in the heat preserved tissue. For the testes tissue, more proteins were detected in the frozen tissue than the heat preserved tissue. It is worth noting that the testes results derive from the sampling of three locations rather than a full image, and this result

may be a consequence of the particular sampling locations. Notably, complementarity in the proteins detected from testes tissue was observed: a total of 86 proteins were detected, with 35 common to both tissue types, 11 unique to heat preserved tissue and 40 unique to frozen tissue.

Methods

Materials

Thin tissue sections: Tissue was obtained from orally dosed and control (vehicle dosed) adult male Hans Wistar rats. Drugs were administered as a cassette comprising olanzapine, terfenadine, nelfinavir and moxifloxacin (kidney) or moxifloxacin, propranolol, clozapine and erlotonib (testes, brain) (all at 10 mg/kg from an oral solution of 5% DMSO, 95% sulfobutyl ether β -cyclodextrin (30% v/v) in water). Animals were euthanized by cardiac puncture under isoflurane anaesthetic 2 hours post dose. All tissue dissection was performed by trained AstraZeneca staff (project licence 40/3484, procedure number 10).

Tissues were either heat-treated (45s at 95°C) using a Stabilisor T1 (Denator AB, Uppsala, Sweden) followed by storage at -80°C or snap frozen in dry-ice chilled isopentane directly after necropsy. Tissues were subsequently cryosectioned at a thickness of 10 μ m and thaw mounted onto glass slides.

Solvents: The following solvents were used: acetonitrile, HPLC grade water (both J. T. Baker, The Netherlands), ethanol (Fisher Scientific, Loughborough, UK), and formic acid (Sigma-Aldrich Company Ltd., Dorset, U.K.).

LESA

Rat brain sections were pre-washed in 70 % ethanol for 10 seconds (to remove abundant lipid species) before air drying and loading onto a universal LESA adapter plate. Thin tissue sections of testes and kidney tissue were not pre-washed. The plate was placed into the TriVersa Nanomate chip-based electrospray device (Advion, Ithaca, NY) coupled to the Thermo Fisher Scientific Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany). An extraction solvent system comprising 40:60 acetonitrile:water with the addition of 1% formic acid was used.

LESA & LESA FAIMS MS, MSI and MS/MS Experiments: 1.5 μ L (testes) or 2.0 μ L (kidney, brain) were aspirated from the solvent well before the robotic arm relocated above the surface of the tissue sample. 1.0 μ L (testes) or 1.5 μ L (kidney, brain) of the solution was dispensed onto the sample surface, forming a liquid microjunction that was maintained for 10 s or 6s (for MSI experiments), before 1.5 μ L or 2.0 μ L was reaspirated. Samples were mixed twice when sampling kidney tissue.

Samples were introduced into the mass spectrometer via the TriVersa NanoMate, with gas pressure 0.3 psi, a tip voltage of 1.75 kV, and a capillary temperature of either 250 °C (no FAIMS) or 350 °C (FAIMS). All MSI experiments were acquired at 2 mm x 2 mm spacing.

FAIMS

The Triversa Nanomate was coupled to a miniaturised ultra-FAIMS device (Owlstone, Cambridge, UK) which was coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen Germany). The FAIMS device was operated either in 2D, static or multi-step static mode. In 2D mode, FAIMS separation was conducted at eight discrete dispersion fields (DFs) between 130 and 270 Td with a step size of 20 Td. At each DF, the compensation field (CF) was varied between -1 to +3 Td over a time period of 180 s. In static mode, the dispersion field (DF) was 270 Td for each experiment, and the CF was either 2.5 Td or 3.0 Td (rat kidney) or either 2.6 Td or 3.0 Td (rat testes). In multi-step static mode, the DF was constant at 270 Td and the CF was held at 2.5 Td for 30 s before switching to 3.0 Td for 45 s.

Mass spectrometry

Experiments were performed on a Thermo Fisher Orbitrap Elite mass spectrometer. Mass spectra were recorded in full scan mode at a resolution of 120 000 at m/z 200 in the m/z range 150-2000 for brain tissue and 200-2000 for kidney and testes tissue. The AGC target was 1×10^6 charges with a maximum injection time of 1000 ms. Automatic gain control (AGC) was turned off for MSI experiments of rat kidney tissue. The fill time was optimised prior to analysis (by sampling serial tissue sections at a central location) by acquiring data with the AGC on with extended maximum injection times. Subsequent interrogation of those data revealed the actual approximate fill times required to accumulate 1×10^6 charges. The following fill times were optimal: 1 ms for LESA experiments, 80 ms for LESA FAIMS experiments on rat kidney at DF = 270 Td, CF = 2.5 Td and 600 ms for LESA FAIMS experiments at DF = 270 Td, CF = 3.0 Td. Data were acquired for 1.25 mins at each location.

MS/MS: Tandem mass spectrometry experiments of LESA-extracted proteins from rat brain (in the absence of FAIMS) and rat testes (DF = 270 Td, CF = 2.6 Td) were conducted via collision induced dissociation (CID). For these experiments, AGC was used with a target of 1×10^6 charges and a maximum injection time of 1000 ms. CID was performed in the ion trap at a normalised collision energy between 25-35% and fragments were detected in the orbitrap. The isolation width was between 3.0-5.0 Th. Each scan comprised of 1 microscan. Data were recorded for between 2-7 minutes (~245-475 scans).

Data Analysis

Protein identification: Data were analysed using Xcalibur version 3.0.63 software. All mass spectra were deconvoluted using the Xtract function in Xcalibur in order to obtain monoisotopic masses of species detected in each experiment. Mass spectra were processed with a signal to noise ratio of 2. To assign fragmentation spectra, .RAW files were passed through the THRASH algorithm in ProSightPC 4.1 Alpha and searched against the UniProt database (Organism: *Rattus norvegicus*, retrieved: 23/01/2018) including all available posttranscriptional modifications, cleavage of initial methionines and N-terminal acetylation, with a maximum of 13 features per sequence. Proteins up to 70 kDa were included in the database. For each MS/MS spectrum, an absolute mass (± 1 kDa) search of the database was initially performed to give a putative assignment, using Δm and disulfide options, and taking into account all available post transcriptional modifications. This broad search used a fragment tolerance of 10 ppm. Assignments were then confirmed by manual analysis and in house software.

Imaging: Single location .raw data files were converted to .mzML using MS convert and then converted to the imzML format and loaded into MATLAB (version 2013a, The MathWorks Inc., Natick, Massachusetts) using imzMLConverter and SpectralAnalysis software⁴⁸. FAIMS: 2D experiments were analysed using in house software as described in ⁴⁹.

Results

LESA FAIMS MS Imaging of Control Rat Kidney

LESA mass spectrometry of fresh frozen and heat preserved tissue sections of control rat kidney, in the absence of FAIMS led to the detection of abundant α - and β -globin ions (~ 16 kDa), see

Supplemental Figure 1. Ion images showing the spatial distribution of 16+ ions of α - and β -globin obtained following LESA mass spectrometry imaging of heat preserved rat kidney tissue are also shown. As expected, these protein species were relatively homogeneously distributed across the kidney tissue. No other protein species were detected in the LESA experiments of frozen tissue; however, β thymosin 4 (4961 Da), ubiquitin (8560 Da) and 10 kDa heat shock protein (10806 Da) were also detected in the heat preserved samples.

Before embarking on a LESA FAIMS mass spectrometry imaging experiment, a two-dimensional FAIMS analysis must be carried out to determine the optimum FAIMS conditions for transmission of the analytes of interest.^{33, 37} **Figure 1A** shows the total ion chromatogram obtained at the discrete DF

step DF = 270 Td during the 2D FAIMS analysis of heat preserved rat kidney. This chromatogram is consistent with previous LESA FAIMS results³³ which suggest the peak centred at CF ~ 0 Td corresponds to high molecular weight unresolved proteins, whereas the peak centered at CF ~ 2.4 Td corresponds to lower molecular weight well-resolved proteins. If optimum transmission of total protein ions was the primary criterion for selection of FAIMS parameters, then based on this chromatogram, a CF of 2-4-2.5 Td would be applied for subsequent LESA FAIMS mass spectrometry imaging. The mass spectra obtained at CF = 2.5 Td and CF = 3.0 Td are shown inset. Mass spectra obtained under static FAIMS conditions are shown in Figure 1B and 1C. Protein species corresponding to α - and β - globin, and 10 kDa heat shock protein, were detected at DF = 270 Td, CF = 2.5 Td from both the heat preserved tissue (Fig 1 B) and frozen tissue (data not shown). Additionally, ubiquitin was detected in the heat preserved sample at this compensation field. At the higher compensation field (3.0 Td) a much wider range of proteins were detected. Figure 1C shows an example mass spectrum from a tissue location in the mass spectrometry imaging data set of heat preserved rat kidney tissue (see below). In this particular tissue location, 20 different protein species were detected in the range 4-15 kDa. That is, in order to maximise the number of proteins detected, multiple FAIMS steps are required.

Based on these results, LESA multi-step static FAIMS mass spectrometry imaging experiments were subsequently performed as follows: at each location, data were acquired for 75 s, first transmitting species at DF=270 Td and CF=2.5 Td for 30 seconds (high abundance protein ions), and then at DF=270 Td and CF=3.0 Td for 45 seconds (lower abundance protein ions). The total imaging analysis time was ~2 hours. Protein species with intact masses 8560 Da (ubiquitin) and 10806 Da (10 kDa heat shock protein), in addition to abundant α - and β - globin ions, were detected at the lower compensation field (2.5 Td) in both the fresh frozen tissue and the heat-preserved tissue. Detection of these additional proteins in the frozen sample (i.e., compared to LESA experiments without FAIMS) is due to the reduced background chemical noise afforded by the inclusion of FAIMS separation. **Supplemental Figure 2** shows the spatial distribution of the α - and β - globin species, in good agreement with the LESA MS imaging data.

Proteins detected in the LESA multistep FAIMS imaging experiments are summarised in **Supplemental Table 1**. Proteins were assigned on the basis of molecular weight, in some cases supported by MS/MS data from other tissues, (see below for further details of protein identification). A total of 37 proteins were detected in the imaging dataset for the fresh frozen tissue section and 59 were detected across the heat preserved sample in the mass range 4-16 kDa. At the higher compensation field (3.0 Td), a total of 31 proteins were detected in the fresh frozen section

and 52 proteins were detected in the heat preserved section. Overall, 61 proteins were detected, with 26 proteins detected in both the fresh frozen and heat-preserved tissue. **Figure 2** shows the spatial distributions of a selection of different protein species detected in heat preserved rat kidney tissue at DF=270 Td and CF= 3.0 Td. Ion images of a selection of proteins from the fresh frozen tissue are shown in **Supplemental Figure 3**. As mentioned above, hemoglobin is highly abundant in kidney tissue and this can present a challenge for mass spectrometry imaging. For example, Herring et al. describe washing of kidney tissue to remove excess hemoglobin prior to MALDI imaging in order to detect protein species that would otherwise be ion suppressed.⁴⁴ The LESA multi-step FAIMS imaging approach enables imaging of less abundant and smaller protein species (transmitted at the higher compensation field) in kidneys without the requirement for sample washing as the ion mobility device affords molecular separation.

Some of the protein species were detected homogeneously across the tissue, for instance ubiquitin (Figure 2F) whereas others were detected in greater intensity around the outer cortex of the kidney, such as the cytochrome c oxidase subunits (Figures 2A-C, 2E and 2H-I). Proteins including ubiquitin (see Figure 2F), acyl Co-A binding protein and a number of cytochrome c oxidases (see Figure 2K, A, B, C, E, H and I) species were assigned by alignment of accurate mass measurements with dissociation data from other tissue types (see below for further details of protein identification). Many of these abundant proteins have been previously reported in rat kidney tissue in MALDI experiments⁵⁰.

We have previously reported separation of phospholipid ions from highly abundant α - and β - globin protein ions via LESA FAIMS of dried blood spots⁵¹. The benefits of FAIMS for separation of abundant α - and β - globin protein ions from less abundant protein ions are shown here. In addition, a number of singly-charged lipid ions were detected at the higher compensation field (3.0 Td) that were not detected either in the LESA experiment in the absence of FAIMS, or with the lower compensation field (2.5 Td), see **Supplemental Fig 4**. Peaks at m/z 758.57, 760.58 and 782.57 are putatively assigned to protonated phospholipid ions (PC 34:2 or PE 37:2 m/z_{calc} = 758.57; PC 34:1 or PE 37:1 m/z_{calc} = 760.58; and PC 36:1 or PE 39:1 m/z_{calc} = 782.57). In the absence of fragmentation data, it is not possible to unambiguously assign these isomeric lipids; however PC species have been more commonly reported (they are the most common lipid in mammalian cells).⁵²

LESA FAIMS MS Imaging of Cassette-Dosed Rat Kidney

LESA mass spectrometry imaging and LESA multi-step FAIMS mass spectrometry imaging of thin tissue sections of frozen and heat-preserved cassette-dosed rat kidney (2 hours post dose) was performed. As seen for the control sample, abundant α -globin and β -globin ions were observed in

frozen tissue samples. **Supplemental Figure 5** shows ion images obtained from the heat preserved tissue following LESA in the absence of FAIMS. α -globin and β -globin, as well as ubiquitin (8560 Da), β -thymosin 4 (4961 Da), heat shock protein (10806 Da) and acyl-CoA binding protein (9932 Da) were detected in the heat preserved samples. In addition, signals corresponding to the drug compounds were detected. (Note, that fexofenadine is the active form of terfenadine). LESA mass spectrometry imaging of three of these drugs (moxifloxacin, terfenadine and olanzapine) in rat kidney has previously been performed.⁴ The spatial distributions observed here are in agreement with those results, demonstrating that drug distribution is unaffected by heat preservation of the tissue.

As seen for the control tissue, increased numbers of proteins were observed following multi-step FAIMS analysis, see **Supplemental Table 1**. A total of 40 proteins were detected across the heat preserved tissue section and 27 were detected across the frozen sample in the mass range 4-16 kDa. At the higher compensation field (3.0 Td), a total of 18 protein species were detected across the frozen tissue sample and 32 protein species were detected across the heat-preserved tissue section. Overall 39 proteins were detected, with 20 proteins detected in both the fresh frozen and heat-preserved tissue. At the lower compensation field (2.5 Td), 8 and 9 proteins, including α - and β -globin were detected in the heat preserved and fresh frozen sections respectively. Three of the four drugs (olanzapine, moxifloxacin and the primary metabolite of terfenadine) were detected in low abundance at both compensation fields. Note that in this work the FAIMS parameters have been optimised for transmission of proteins therefore detection of the drug compounds is not necessarily expected. The multi-step FAIMS approach could in principle be adapted for any combination of molecular classes, e.g., a FAIMS step optimised for transmission of small molecule drugs and a step optimised for transmission of proteins. Moreover, the only limitations of the number of FAIMS steps are the length of time the electrospray signal lasts following LESA sampling (typically ~ 20min, maximum achieved in our hands ~60 mins), the required number of co-added mass spectra to achieve sufficient spectra quality, the resolving power of the FAIMS device, and the time taken to acquire the imaging dataset. Representative ion images obtained from the heat preserved tissue are shown in **Supplemental Figure 6**.

LESA and LESA FAIMS MS of Cassette-Dosed Rat Testes

To further investigate the benefits of both heat preservation of tissue and multi-step static FAIMS analyses, experiments were performed on fresh frozen and heat preserved tissue sections of cassette-dosed rat testes. For each sample type, three locations were sampled by LESA and the combined results are described. LESA mass spectrometry (in the absence of FAIMS) of frozen testes tissue led to the detection of 5 protein species, see **Supplemental Table 1**, whereas sampling of heat

preserved testes tissue led to the detection of 20 proteins. Example LESA mass spectra of heat preserved and frozen tissue are provided in **Figure 3A** and **3B**. Four of the five proteins detected in the frozen sample (ubiquitin, β -thymosin 4, ATP synthase subunit e, and cytochrome C oxidase 7C) were also detected in the heat preserved sample, while one (ATP synthase ϵ) was not. Of the 16 unique protein species detected in the heat preserved sample, 11 were detected in the frozen sample once FAIMS was integrated into the workflow (see below). Similarly, the one unique protein detected in the frozen sample was detected in the heat preserved sample with the implementation of FAIMS.

Prior to performing static FAIMS experiments, a 2D FAIMS analysis was performed to identify the optimum DF and CF parameters for transmission of proteins extracted from testes tissue. As with the 2D FAIMS analysis of the kidney samples, it was found that at higher dispersion fields there was greater separation between proteins and other molecular species. At DF = 270 Td and CF = 2.6 Td, higher molecular weight proteins such as myelin basic protein and α - and β - globin were transmitted, whereas at DF = 270 Td and CF = 3.0 Td lower molecular weight proteins, such as acyl-CoA binding protein and ATP synthase ϵ , were transmitted.

Figure 3C-F show representative static FAIMS mass spectra obtained following LESA sampling of rat testes. Proteins detected in these experiments are summarised in **Supplemental Table 1**. Proteins were assigned on the basis of molecular weight, and MS/MS where indicated. A total of 75 proteins were detected in the frozen tissue and 46 were detected in the heat preserved tissue in the mass range 4-16 kDa. For the frozen tissue, 42 proteins were detected at CF = 3.0 Td (Figure 3F) and 39 were detected at 2.6 Td (Figure 3D). The additional protein species detected in the heat preserved tissue sample in the absence of FAIMS were also detected in the frozen sample when FAIMS was incorporated into the workflow (static experiments at DF = 270 Td and CF = 3.0 Td), likely due to the improved S/N associated with FAIMS. For the heat preserved tissue, 28 proteins were detected at CF = 3.0 Td (Figure 3E) and 15 were detected at CF = 2.6 Td (Figure 3C). That is, fewer proteins were detected following LESA FAIMS analysis of heat preserved tissue compared with frozen tissue, which may reflect the smaller sample set for testes tissue, i.e., the numbers reported are the proteins detected in at any of the three locations sampled, rather than across an entire image as for the kidney samples. The proteins detected following LESA MS or LESA FAIMS MS of heat preserved and frozen testes tissue samples are summarised in Figures 3G and 3I respectively. The number of proteins detected from the two tissue types across all experiments are shown in Figure 3H. Overall, 86 proteins were detected, with 35 proteins detected in both the fresh frozen and heat preserved tissue.

Protein identification by MS/MS

Proteins observed in the LESA and LESA FAIMS mass spectra were either putatively assigned based on their intact mass, or assigned by collision-induced dissociation, or a combination of the two. For example, the protein ATP synthase ϵ observed in rat kidney was assigned based on its intact mass and MS/MS data obtained following LESA FAIMS analysis of rat testes. The origin of assignments is detailed in **Supplemental Table 1**. In total, 13 proteins were identified following LESA CID MS/MS or LESA FAIMS CID MS/MS. Seven proteins were identified from rat testes (ubiquitin (8560 Da); cytochrome C oxidase 7A2 (6644 Da); myelin basic protein isoform 4 (14112 Da); ATP synthase ϵ (5632 Da), acyl-CoA binding protein (9932 Da), ATP synthase coupling factor 6 (8922 Da) and cytochrome C oxidase 6C2 (8360 Da). Three of the proteins identified, cytochrome C oxidase 7A2, acyl-CoA binding protein and ATP synthase coupling factor 6, were not observed by LESA alone, but the incorporation of FAIMS into the workflow allowed these proteins to be detected in sufficient abundance that it was possible to obtain MS/MS data. Nine proteins extracted from rat brain tissue were subjected to CID fragmentation, three of which had also been identified by MS/MS from testes tissue. The remaining six proteins were identified as β -thymosin 10 (4934 Da), cytochrome c oxidase 7C (5482 Da), cytochrome c oxidase 6C-2 (8360 Da), 10 kDa heat shock protein (10806 Da) macrophage migration inhibitory factor (12360 Da) and calmodulin (16780 Da). All proteins identified by MS/MS from brain, with the exception of macrophage migration inhibitory factor and calmodulin, were subsequently used to assign the identity of proteins with matching intact masses in rat kidney or testes tissue samples. Fragment assignments and sequence coverages are given in **Supplemental File 2**.

Conclusions

We have demonstrated an improved LESA FAIMS mass spectrometry imaging workflow, in which LESA extraction at each individual location is coupled with multi-step static FAIMS separation. This approach significantly improves the numbers of proteins that can be detected and imaged. The approach was applied to protein imaging in kidney sections, which are typically dominated by α - and β -globin, and enabled the detection of up to 59 proteins when multi-step FAIMS was incorporated into the imaging workflow. Similar improvements were observed when multi-step FAIMS was coupled with LESA mass spectrometry of testes tissue, in which up to 75 proteins were detected.

We have also demonstrated that heat preserved tissue is suitable for LESA (FAIMS) mass spectrometry imaging. For the kidney tissue, an $\sim 1.5\times$ improvement in the number of proteins detected was observed when heat treated tissue was compared with frozen tissue. For the testes

tissue, fewer overall proteins were identified in the heat treated tissue than the frozen tissue but greater complementarity between the two tissue types was observed.

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Figures:

Figure 1. LESA FAIMS MS of heat preserved rat kidney tissue. A) Total ion chromatogram obtained at DF = 270 Td step during 2D FAIMS analysis. Mass spectra at CF = 2.5 Td and 3.0 Td are shown inset. Mass spectra obtained under static conditions: B) DF = 270 Td, CF = 2.5 Td and C) DF = 270 Td, CF = 3.0 Td. Static FAIMS data were taken from imaging dataset (pixel 32).

Figure 2. LESA FAIMS MS molecular ion images of proteins detected in heat preserved rat kidney tissue (DF = 270 Td, CF = 3.0 Td). Stated m/z is the mid-point of the isotopic distribution of the protein species indicated, ion images were produced from the most abundant peak in the isotope distribution. Molecular ion images of A) m/z 985.5 (5+)*, B) m/z 915.2 (6+), C) m/z 1109.1 (6+), D) m/z 1016.3 (8+)*, E) m/z 930.5 (9+), F) m/z 857.5 (10+), G) m/z 992.8 (9+), H) m/z 933.6 (10+), I) m/z 967.5 (10+)*, J) m/z 884.5 (11+), K) m/z 829.2 (12+), L) m/z 829.4 (12+), M) m/z 832.8 (13+), N) m/z 867.5 (14+) and O) 1169.9 (13+)* were detected in both frozen and heat treated tissue samples. P) m/z 925.8 (9+), Q) m/z 661.6 (14+), R) m/z 865.2 (8+), S) m/z 842.8 (17+) and T) m/z 897.5 (7+) were unique to the heat treated sample. *denotes proteins assigned according to measured intact mass.

Figure 3. Example LESA mass spectra from A) heat preserved and B) frozen testes tissue and LESA FAIMS mass spectra (DF = 270 Td, CF=2.6 Td) from C) heat preserved and D) frozen testes tissue and at DF = 270 Td, CF=3.0 Td from E) heat preserved and F) frozen testes tissue. Venn diagrams comparing proteins detected from G) heat preserved testes tissue using LESA MS and LESA FAIMS MS (DF = 270 Td, CF = 2.6 Td and DF = 270 Td, CF=3.0 Td H) heat preserved and frozen testes samples across all experiments and I) frozen testes tissue using LESA MS and LESA FAIMS MS (DF = 270 Td, CF = 2.6 Td and DF = 270 Td, CF = 3.0 Td).

TOC Graphic:

